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MITOCHONDRIAL PREPARATIONS FROM THE FRUIT OF THE APPLE—II.

OXIDATIVE PHOSPHORYLATION

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Abstract—Mitochondria prepared from the peel and pulp of apple fruits gave good oxidative phosphorylation with acids of the Krebs' cycle. They also exhibited the phenomenon of "respiratory control". In senescent fruit the power of the mitochondria prepared therefrom to fix inorganic phosphate decreases and respiratory control is also much decreased. There are indications that loss of phosphorylation and respiratory control by mitochondria prepared from senescent fruits may be overcome to some extent by the incorporation of bovine serum albumin in the extraction medium.

THE WORK of Pierpoint[1] on tobacco leaf and Hackett *et al.*[2] on sweet potatoes may be taken as representative of experiments to examine the capacity for oxidative phosphorylation of mitochondrial preparations from plant sources. Maximum P:O ratios obtained by Pierpoint for the overall reactions with various Krebs' cycle substrates were about half the assumed theoretical values,[3] and ranged from 1·3 for succinate as substrate to 2·1 for isocitrate. Hackett obtained appreciably higher values. Lieberman[4] showed that cytoplasmic particles prepared from apple pulp by his alkaline extraction methods effect oxidative phosphorylation with α-oxoglutarate and succinate as substrate. His P:O ratios were, however, low; for the overall reactions with α-oxoglutarate the value was 0·9, and for the single step with this acid (i.e. in presence of malonate) the value was 1·3.

Respiratory control (by ADP) has been fairly extensively studied in animal mitochondria (e.g. Chance and Williams[5]), but Bonner and Voss[6] were the first to show that plant mitochondria may exhibit the phenomenon of respiratory control with, in some cases, practically no O_2-uptake in the presence of substrate in the absence of ADP. Although the work of Hoch and Lipmann,[7] Chance and Williams,[5] and Baltscheffsky[8] on respiratory control and oxidative phosphorylation in mitochondria from animal sources indicates that the two phenomena are not necessarily different aspects of a single system, it is clear that both are intimately concerned in the provision of energy, through the high energy phosphate bond, by the respiratory processes.

Dalgarno and Birt[9] have recently shown that free fatty acids can act as uncouplers of oxidation and phosphorylation. In mitochondria from carrots, this uncoupling can be

[1] W. S. PIERPOINT, *Biochem. J.* 75, 504 (1960).
[2] D. P. HACKETT, B. RICE and C. SCHMID, *J. Biol. Chem.* 235, 2140 (1960).
[3] H. A. KREBS, *Chemical Pathways of Metabolism*, Vol. 1, p. 162, Academic Press, New York (1954).
[4] M. LIEBERMAN, *Plant Physiol.* 36, 804 (1961).
[5] BRITTON CHANCE and G. R. WILLIAMS, *J. Biol. Chem.* 217, 383 (1955).
[6] W. D. BONNER and D. O. VOSS, *Nature, Lond.* 191, 682 (1961).
[7] F. L. HOCH and F. LIPMANN, *Proc. Nat. Acad. Sci. Wash.* 40, 909 (1954).
[8] H. BALTSCHEFFSKY, *Biochem. et Biophys. Acta* 25, 382 (1957).
[9] L. DALGARNO and L. M. BIRT, *Biochem. J.* 87, 586 (1963).

reduced by bovine plasma albumin. It has long been known that plasma albumin has the capacity to bind free fatty acids and Goodman[10] has recently made a careful study of this problem in relation to long-chain fatty acids. The object of the present work was to investigate phosphorylation by apple mitochondria at two stages of storage and to seek evidence for respiratory control.

RESULTS

Oxidative Phosphorylation

Pierpoint[1] in his work on phosphorylation used as co-factors a mixture of ATP, NAD, thiamine pyrophosphate and CoA. Since for various reasons we wished to carry out extensive experiments on phosphorylation by apple mitochondria it was worth investigating, on the

TABLE 1. UPTAKE OF INORGANIC PHOSPHATE (P_i) AND O_2, AND P:O RATIOS OF MITOCHONDRIA FROM APPLE PEEL WITH SUCCINATE AND ISO-CITRATE AS SUBSTRATE (SERIES A FRUIT)

Substrate	Time from equilibration (min)	Co-factors: mixture (MX)			Yeast concentrate (Y)		
		P_i (μ atoms)	O_2	P:O	P_i (μ atoms)	O_2	P:O
Succinate (0·02 M)	30	8·5	5·5	1·55	10·7	6·3	1·70
	60	14·2	9·1	1·56	17·1	11·7	1·46
DL-iso-citrate (0·04 M)	30	4·5	2·1	2·14	4·6	2·2	2·10
	60	7·4	4·4	1·68	7·8	5·4	1·45

grounds of economy, the effect of replacing this mixture (MX) by concentrated yeast extract (Y) which should contain all the ingredients of this mixture and possibly other factors in addition. The results given in Tables 1 and 3 show that, although both O_2-uptake and P_i-uptake are generally somewhat higher with yeast concentrate, the P:O ratios show no definite

TABLE 2. CHANGE IN P:O RATIOS WITH TIME FOR MITOCHONDRIA FROM APPLE PEEL PREPARED WITH AND WITHOUT PVP IN THE EXTRACTION MEDIUM (SERIES A FRUIT—SUCCINATE AS SUBSTRATE)

Time (min)	Preparation 1 (MX) prepared in presence of 4% PVP			Preparation 2 (Y) prepared in presence of 4% PVP			Preparation 3 (Y) prepared in absence of PVP		
	P_i	O_2 (μ atoms)	P:O	P_i	O_2 (μ atoms)	P:O	P_i	O_2 (μ atoms)	P:O
15	4·6	3·0	1·54	3·7	2·6	1·42	0	0·78	0
30	9·9	5·1	1·95	8·1	5·8	1·40	0	1·7	0
45	12·2	7·3	1·67	12·8	9·1	1·41	0	2·7	0
60	12·7	8·0	1·59	17·1	11·8	1·44	0·32	3·2	0·10
75	17·1	11·2	1·53	19·1	13·7	1·39	1·1	4·4	0·25

trend towards either supplement. Figure 1 illustrates the progress with time of O_2-uptake and P_i-uptake with peel mitochondria prepared with 4% PVP in the extraction medium using succinate as substrate; Table 2 shows the P:O ratios for three preparations (preparation 1, 4% PVP; preparation 2, 4% PVP; preparation 3, no PVP in extraction medium) with

[10] D. S. GOODMAN, *J. Am. Chem. Soc.* **80**, 3892 (1958).

succinate as substrate. The almost complete lack of phosphorylation by mitochondria prepared without the incorporation of PVP in the extraction medium (Hulme and Jones [11]), even when some oxidation was taking place, is evident from Table 2. The P:O ratios obtained

TABLE 3. P:O RATIOS WITH VARIOUS SUBSTRATES FOR MITOCHONDRIA PREPARED FROM THE PEEL AND PULP OF APPLES (SERIES A FRUIT)

Substrate (0·02 M)	Other factors	Time from equilibration (min)	O_2-uptake (μ atoms)	P:O
Peel				
citrate	Y	60	1·38	1·99
	MX	60	0·84	3·32
cis-aconitate	Y	30	2·75	2·10
	MX	30	3·45	1·51
α-oxoglutarate	Y	30	3·06	2·32
α-oxoglutarate + malonate	Y	30	1·00	2·89
fumarate	Y	30	1·46	2·23
	MX	30	1·34	2·01
malate	Y	30	6·94	2·61
		60	12·05	1·77
malate minus glucose and hexokinase	Y	30	4·26	1·35
pyruvate plus 2 μmol malate	Y	30	1·27	2·24
		60	2·65	1·95
Pulp				
succinate	Y	60	3·30	1·00
α-oxoglutarate	Y	60	1·29	1·80

with various Krebs' cycle acids are shown in Table 3. Most of the results are for peel mitochondria but the two results available for a pulp preparation made from Series A fruit stored for one month longer than the results for peel show that these mitochondria also fix

TABLE 4. THE EFFECT OF DNP ON OXIDATIVE PHOSPHORYLATION BY MITOCHONDRIA FROM APPLE PEEL; 0·02 M MALATE SUBSTRATE, 30-MIN PERIOD FROM EQUILIBRATION (SERIES A FRUIT)

Concentration of DNP, $M \times 10^{-5}$	P_i (μ atoms)	O_2	P:O
0	15·0	5·1	2·94
5	8·1	4·9	1·65
10	9·1	4·9	1·85
50	0	0·12	0

phosphate. The data given in Table 4 show that at relatively high concentrations dinitrophenol (DNP) inhibits completely both oxidation and phosphorylation, but at lower concentrations (1×10^{-4} and 5×10^{5} M) it uncouples phosphorylation from O_2-uptake.

[11] A. C. HULME and J. D. JONES, *Enzyme Chemistry of Phenolic Compounds*, p. 97, Pergamon Press, London (1963).

Respiratory Control

The ideal method for investigating "respiratory control" is with the oxygen electrode which has high sensitivity and quick response (Bonner and Voss[6]). Nevertheless, Hackett *et al.*[2] have demonstrated some measure of respiratory control by ADP of the activity of mitochondria from the sweet potato using the Warburg respirometer technique. Since an oxygen electrode was not available, all our measurements of O_2-uptake were made in a Warburg respirometer. The results obtained with stored apples in good condition (i.e. picked in the pre-climacteric state and stored in air for three months at 3° C, Series A) indicate, that

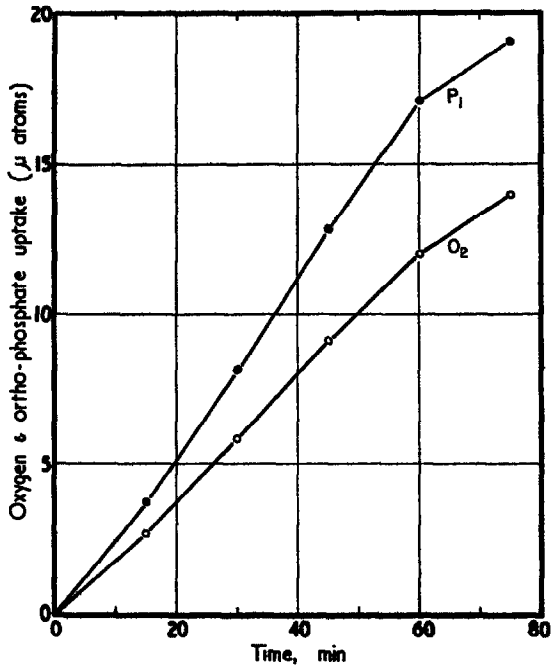

FIG. 1. O_2-UPTAKE AND P_i-UPTAKE OF MITOCHONDRIA FROM THE PEEL OF APPLES (SERIES A), WITH SUCCINATE AS SUBSTRATE.

with NADH as substrate, the mitochondria prepared from the peel showed an increased O_2-uptake when ADP was added, and this stimulation decreased fairly rapidly (Fig. 2). This was also true to a more limited extent with α-oxoglutarate as substrate. With both substrates the rate of respiration without addition of ADP slowly increases to an approximately constant rate. The simplest explanation of this fact is that the mitochondria contain some ADP and some ATPase which gives a slow, steady regeneration of the ADP. As time proceeds the mitochondria "age" and this ATPase activity may be increasing (Stumpf[12]). Assuming an actual P:O ratio for α-oxoglutarate of 2·4 (see Table 3) 10 μmol of ADP would require only 4·5 μ atoms, or approximately 55 μl of O_2, for its complete utilization. This should have occurred about 2 hr after the addition of ADP (Fig. 2, α-oxoglutarate). Clearly there must have been some regeneration of ADP. The increased O_2-uptake in the absence of ADP shown in Fig. 3 for mitochondria from the more senescent fruit of Series B would, on this

[12] P. K. STUMPF, *Chemical Pathways of Metabolism*, Vol. 1, p. 67, Academic Press, New York (1954).

argument, appear to be due to an increased activity of ATPase. In general there is some lag in the response to added ADP as mentioned by Bonner and Voss.[6] It is, no doubt, not well marked here because of the inevitably longer time-interval measurements.

The present consensus of opinion (see Klingenberg and Bücher[13]) is that the preferred method of calculating the respiratory control ratio (R.C. ratio) is to divide the maximum rate of O_2-uptake in presence of added ADP by the rate when all the ADP has been used up. This method cannot be used here partly because of the slow response of the Warburg technique but mainly, we suggest, because of the presence of ATPase in our preparations. That respiratory control is present is quite clear from the results with NADH as substrate with the

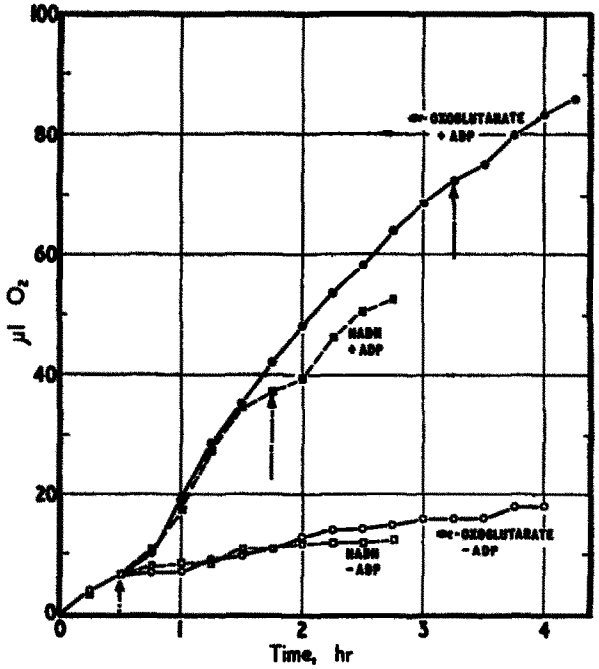

FIG. 2. O_2-UPTAKE OF MITOCHONDRIA FROM THE PEEL OF APPLES (SERIES A) WITH α-OXOGLUTARATE AND WITH NADH AS SUBSTRATES.

The effect of adding 10 µmol ADP at each of the points marked by arrows. Open symbols, no ADP present; filled-in symbols, ADP present.

mitochondria from less senescent tissue (Fig. 2). The suggestion of Bonner and Voss[6] that the failure of the O_2-uptake to fall to a steady low state when the ADP is all used up is due, in plant mitochondria, to a factor present in the starch fraction cannot be valid here. There is no starch left in the apples at this stage and our mitochondrial preparations show no sign of starch or any starch-like fraction. In this preliminary work on "respiratory control" in apple mitochondria we have, for purposes of comparisons between the two sets of apples in different states of senescence, calculated the R.C. ratios as follows: The O_2-uptake per 15 min over the period 15–45 min after the addition of ADP is divided by the corresponding O_2-uptake of the sample to which ADP has not been added. This eliminates the effect of the lag period.

[13] M. KLINGENBERG and T. BÜCHER, *Ann. Rev. Biochem.* **29**, 669 (1960).

Changes in Phosphorylation and Respiratory Control with Increasing Senescence

The fruit of Series B was very senescent and, although free from fungal attack and physiological disease, it was soft and tasteless.

It should be remembered (see Experimental section, "Respiratory Control") that phosphorylation with mitochondria from these fruits was measured at the same time as "respiratory control", i.e. in the Warburg flasks; glucose, hexokinase and sodium fluoride (NaF)

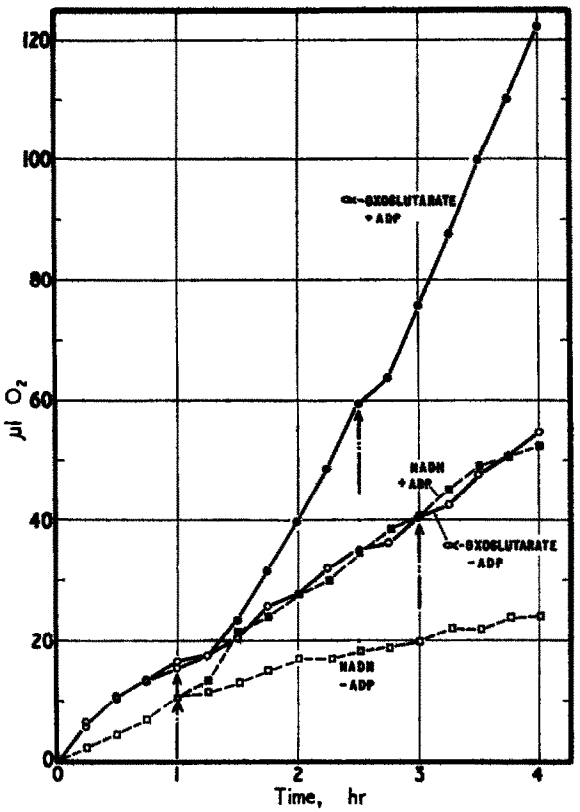

FIG. 3. O_2-UPTAKE OF MITOCHONDRIA FROM THE PULP OF APPLES (SERIES B) WITH α-OXOGLUTARATE AND WITH NADH AS SUBSTRATE.

2 μmol ADP added at each of the points marked by an arrow. Open symbols, no ADP; filled-in symbols, ADP present.

were absent and yeast extract was replaced by NAD alone. The only phosphate acceptor present was ADP, so that in the presence of active ATPase, measured P:O ratios would be expected to be low owing to the regeneration of P_i. The influence of bovine plasma albumin will be discussed below.

The *observed* P:O ratios from the senescent fruit (Series B) are very much lower than those for the less senescent fruit (Series A). The corresponding average values at 60 min for peel with α-oxoglutarate as substrate are 2·32 and 0·66, and for pulp 1·80 and 0·52 (from Tables 3 and 5). In spite of the reservations mentioned above it is very probable that the *true* P:O ratios for fruit of Series B are considerably lower than those for fruit of Series A. Support

for this argument comes from the results shown in Fig. 4 (Curve 2) for peel, where NaF is present throughout the experiment to minimize ATPase activity; the P:O ratio here, with α-oxoglutarate, was 1·25. Again, where glucose and hexokinase were present in sufficient

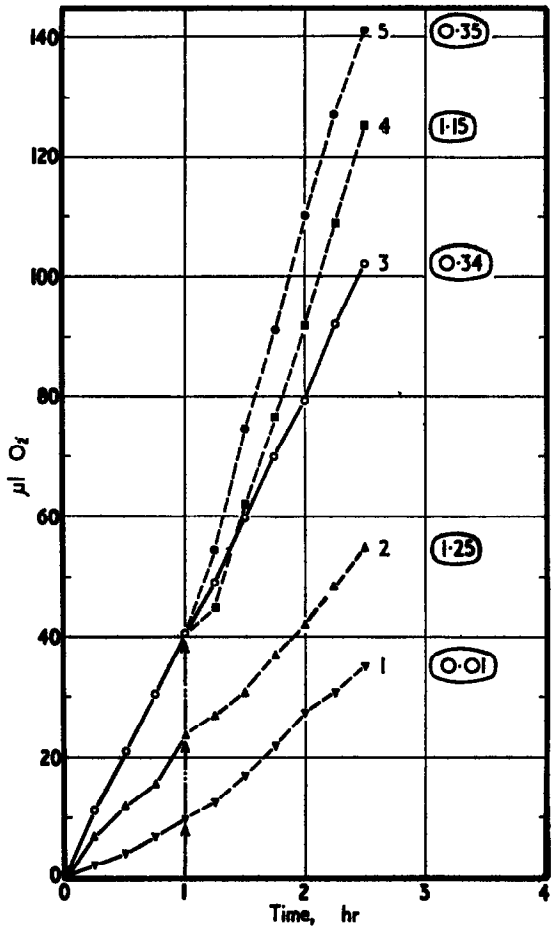

FIG. 4. O_2-UPTAKE OF MITOCHONDRIA FROM THE PEEL OF APPLES (SERIES B—BOVINE PLASMA ALBUMIN ADDED DURING PREPARATION) WITH α-OXOGLUTARATE AS SUBSTRATE.

Curve 1, Mg^{2+} absent throughout, 2 μmol ADP added at arrow (▼). Curve 2, 15 μmol NaF present throughout, 2 μmol ADP added at arrow (▲). Curve 3, no ADP present (○). Curve 4, no ADP, but 20 μmol glucose and 53 units hexokinase added at arrow (■). Curve 5, 2 μmol ADP added at arrow (●). The values inside circles to the right of each curve represent the P:O ratios between 1 and 2½ hr from zero time.

quantity to "swamp" ATPase activity and provide phosphate acceptor (Curve 4) the P:O ratio was still only 1·15. The data for "respiratory control" as defined above are shown in Table 6. Neglecting for the moment the effect of the addition of bovine plasma albumin during the preparation of the mitochondria, it is evident that the R.C. ratios are very much lower for the more senescent fruit.

Baltscheffsky[8] suggests for animal mitochondria that, in a medium free from Mg^{2+}, the

particles rapidly change from the "intact" to the "aged" condition. In Fig. 4, Curve 1, is illustrated the effect of a Mg^{2+} free medium or our peel mitochondria. Since the first reading is 30 min from zero time it is probable that our mitochondria would have been in the "aged"

Table 5. P:O ratios of mitochondria from peel and pulp of very senescent apples (Series B) with α-oxoglutarate as substrate. Mitochondria prepared with and without bovine plasma albumin in extraction media. 2·8 μ-mol ADP added after 60 min to one set of samples

Tissue	Bovine plasma albumin	After 60 min (No ADP) P_i (μ atoms)	O_2	P:O	Between 60 and 150 min (No ADP) P_i (μ atoms)	O_2	P:O	(ADP present) P_i (μ atoms)	O_2	P:O
Pulp	–	1·5	2·7	0·55	0·98	3·5	0·28	3·4	5·6	0·61
	+	0·62	1·3	0·48	0·84	1·5	0·56	2·9	3·6	0·79
Peel	–	2·0	3·1	0·65	2·0	5·2	0·39	1·7	7·5	0·23
	+	2·3	3·4	0·68	1·7	5·0	0·34	2·8	8·1	0·35
Plus Malonate										
Pulp	+	1·5	1·4	1·07	1·2	3·2	0·37	1·2	4·1	0·29
Peel	+	1·7	2·3	0·74	1·4	4·6	0·31	2·4	6·1	0·39

Table 6. R.C. ratios (see text) for mitochondria prepared from apple fruits at two stages of senescence

Tissue	Substrate	O_2-uptake per 15 min (μl–see text) −ADP	+ADP	R/C Value
Series A				
peel, −bov	α-oxoglutarate	1·8	10·4	5·8
peel, −bov	NADH	0·5	8·2	16·4
Series B				
pulp, −bov	α-oxoglutarate	7·5	11·0	1·5
pulp, −bov	NADH	2·0	6·2	3·1
pulp, +bov	α-oxoglutarate	3·0	7·0	2·3
peel, −bov	α-oxoglutarate	12·5	17·0	1·4
peel, +bov	α-oxoglutarate	11·5	18·0	1·6
Series B				
pulp, +bov	α-oxoglutarate	7·0	9·0	1·3
peel, +bov	+malonate	11·0	12·0	1·1

state from the first reading. This hypothesis is supported by the very low rate of respiration, the lack of respiratory control and the virtual absence of phosphorylation (P:O ratio = 0·01).

Finally to consider the effect of adding crystalline bovine plasma albumin (see Experimental section) to the extraction medium when preparing mitochondria from the fruit of Series B. The average P:O ratios (both with and without ADP) minus albumin are 0·51

for pulp and 0·38 for peel (Table 5). With albumin the values are 0·66 for pulp and 0·43 for peel. The R.C. ratios, low though they are, do show some increase when albumin is used.

DISCUSSION

At least the major part of the respiratory O_2-uptake and CO_2-output of fruits must arise from the metabolism of organic acids through the action of the Krebs' cycle enzymes located in the mitochondria. It has been suggested (Hulme *et al.*[14]) that the climateric rise in CO_2-output, accompanied by a smaller rise in O_2-uptake, may be due to the operation of malic enzyme and pyruvic carboxylase. Energy is required for the synthesis of these enzymes as well as other enzymes associated with the ripening process. The energy for the synthesis of this protein may be supplied by the high energy phosphate compound ATP. Merely to measure respiration in terms of O_2-uptake or, and this is technically simpler to achieve, of aerobic production of CO_2, gives little insight into the energetics of the process. If the respiration is not coupled to phosphorylation (i.e. if ATP is not produced during the process) the energy yield will be low, and for any given rate of respiration the development of chemically useful energy can vary within wide limits, depending on the degree of uncoupling. There is, in fact, a tendency for the respiration rate to rise when the respiration becomes uncoupled from phosphate uptake. It is important, therefore, when studying periods of physiological change, such as senescence, to find out how closely respiration is geared to phosphorylation. Owing to the complexity of the total metabolism of a tissue it is simpler to study oxidative phosphorylation at its source, namely the mitochondria. As indicated by the work of Chance and Williams,[5] Hock and Lipmann[7] and others for animal mitochondria the P:O ratio is itself not a very sensitive measure of the tightness of coupling between respiration and phosphorylation. Respiratory control by ADP is a better measure of the degree of coupling. The present results show that in fruit (Series A), which experience over many years suggests is not past the climacteric peak in respiration, the P:O ratios for the mitochondria isolated from them are near to the theoretical values for the sequence of Krebs' cycle acids, especially for the "single stage" oxidation of α-oxoglutarate in presence of malonate. They are as high as those for mitochondria from other plant sources (e.g. Hackett *et al.*[2]). In the much more senescent fruit (Series B), the P:O ratios of the mitochondria prepared from them are very much lower, although the O_2-uptake itself is not greatly reduced. This suggests that at this later stage in senescence, oxidation has to a considerable extent become uncoupled from phosphorylation.

The results, especially those with NADH as substrate, provide evidence of respiratory control in mitochondrial preparations from the fruit in good condition (Series A). The values for the R.C. ratio, calculated by the arbitrary method used here, for α-oxogluturate and NADH as substrates are 5·8 and 16·4 respectively. Calculated by the conventional method used for animal mitochondria (O_2-uptake immediately on addition of ADP divided by the lowest rate reached subsequently), the value obtained for α-oxoglutarate would be 2·5 and for NADH 3·1 (Fig. 2). The R.C. values for the more senescent fruit are very much lower. Although this may be due in part to the apparent increased ATPase activity in the mitochondria prepared from this tissue, the results support the suggestion that coupling between oxidation and phosphorylation is much less tight in the more senescent fruit of Series B than in the fruit of Series A. It is possible that the mitochondria prepared from the more senescent fruit have suffered structural damage; we have as yet no electron micrographs of the mitochondria from this tissue comparable with those from less senescent tissue.[15]

[14] A. C. Hulme, J. D. Jones and L. S. C. Wooltorton, *Proc. Roy. Soc. B.* **158**, 514 (1963).
[15] A. C. Hulme, J. D. Jones and L. S. C. Wooltorton, *Phytochem.* **3**, 173 (1964).

A preliminary attempt has been made to see whether the uncoupling of oxidation and phosphorylation can be attributed to the action of free fatty acids, as suggested by Dalgarno and Birt[9] for mitochondria from carrots (see also Klingenberg and Bücher[13]). Meigh[16] has shown that an appreciable part of the wax of the cuticle and epidermis of stored apples consists of C_{16} and C_{18} fatty acids, although the degree of esterification *in vivo* is not known. It is possible, therefore, that fatty acids in the tissue itself could be liberated during the preparation of the mitochondria and could become involved in such an uncoupling process. The data here obtained is inconclusive since the addition of bovine plasma albumin to absorb fatty acids during the preparation of the mitochondria, has only a marginal effect on P:O and R.C. ratios. The negligible effect of the addition of albumin to peel tissue may be because the amounts of fatty acids present in this tissue are too great to be absorbed by the amount of albumin used. The results are sufficiently encouraging to warrant a more thorough investigation of the problem, including measurement of changes in lipase activity and free fatty acids during the senescence of apple fruits.

EXPERIMENTAL

Fruit Used

All the apples used were of the Cox's Orange Pippin variety picked at the stage normal for long storage, i.e. mature but just before the onset of the respiration climacteric (see Hulme *et al.*[14]). *Series A* fruit was from 50 trees grown on Malling IX rootstocks in a grassed-down orchard and stored at 3° for 3 months in air. *Series B* fruit was from a good commercial orchard stored in controlled atmosphere storage (0·5% CO_2, 2·5% O_2) at 3° for 8½ months. Experience has shown that there is unlikely to be any fundamental difference in the original fruit of the two series of samples.

Peel tissue was prepared as previously described (Hulme *et al.*[15]) and pulp tissue was taken, in strips as for the peel, from throughout the cortex of the fruit. All these manipulations were done in a room at 1°.

Preparation of Mitochondria

For Series A fruit, the mitochondrial fraction was prepared exactly as described by Hulme *et al.*,[15] using 0·75–4·0% polyvinylpyrrolidone (P.V.P.) in the extraction medium.

Later experiments showed that both citrate and phosphate can be omitted without influencing the activity of the final preparation. These two ingredients were, therefore, omitted from the extraction medium when preparing the mitochondria of Series B fruit. Since the mitochondria of this latter series were less active, 50 g (instead of 25 g) was used in preparing the peel mitochondria and 100 g when preparing the pulp mitochondria. The standard ratio (Hulme *et al.*[15]) of the weight of tissue to the volume of extraction medium was maintained throughout. Since the present paper is only concerned with P:O ratios and respiratory control (R.C.) this difference in "concentration" of mitochondrial suspension is unimportant.

In some experiments in Series B (see Results section) bovine plasma albumin was incorporated in the extraction medium. When this was done, 0·5% (w/v) of the albumin was dissolved in the extraction medium immediately before use. In these cases the medium used to wash the mitochondria contained 0·2% (w/v) albumin, and 11 mg albumin per 5 ml was incorporated in the final suspending medium.

[16] D. F. MEIGH, *J. Sci. Food Agric.* (in press).

Phosphorylation

Uptake of inorganic phosphate was measured by estimating the disappearance of orthophosphate in Warburg manometric flasks using essentially the technique of Pierpoint,[1] but employing the colorimetric method of Allen[17] for the determination of phosphate.

The contents of the Warburg flasks were as follows: Sucrose, 400 μmol; phosphate buffer (pH 7·5), 7·5 to 12·5 μmol depending on period of phosphorylation, substrate and activity of the mitochondrial preparations; $MgSO_4$, 10 μmol; $MnSO_4$, 0·1 μmol; cytochrome C, 0·018 μmol; glucose, 10 μmol; hexokinase, 1 mg; yeast concentrate 1 mg (in some experiments this was replaced by the following mixture dissolved in 0·1 ml water; ATP, 2·0 μmol; NAD, 0·2 μmol; thiamine pyrophosphate, 0·2 μmol; CoA, 0·025 μmol); sodium fluoride 15 μmol; crystalline bovine plasma albumin, 3 mg; acid substrate, 40 μmol; mitochondrial suspension 0·5–1·0 ml. When a "one step" oxidation of α-oxoglutarate was studied, 40 μmol of malonate was also added to the contents of the flask which were always made to a final volume of 2 ml with water.

The flasks were equilibrated in the Warburg bath (at 25°) for 5 min before the manometer taps were closed. A further equilibration period of 10 min was allowed before the first two flasks (replicates) were taken for the determination of "initial" orthophosphate content (P_i). This overall equilibration period of 15 min before the initial P_i content was measured was found to be essential for consistent results. Two more flasks were taken for the determination of P_i (O_2-uptake now measured every 15 min) from time to time as indicated in the tables in the Results section. To measure P_i-uptake, 1 ml of liquid was removed from the Warburg flasks and added immediately to 1 ml of ice cold 5% (w/v) trichloracetic acid in a test tube immersed in an ice bath. After mixing and allowing to stand for 10 min, the tubes were centrifuged for 10 min at 1500 *g*. One ml of the supernatant solution was then added to 4 ml of ice cold 0·1 M sodium acetate solution and an aliquot portion taken for the determination of phosphorus by Allen's[17] method. Standard orthophosphate solutions were incorporated in each set of determinations.

Respiratory Control

Respiratory control was measured by comparing the O_2-uptake with and without the addition of ADP (see Results section, p. 204). The basic digest in the Warburg flasks for the experiments with mitochondria from apples of Series A was as follows: Sucrose, 400 μmol; KCl, 20 μmol; $MgCl_2$, 10 μmol; substrate, 20 μmol; crystalline bovine plasma albumin, 3 mg; phosphate buffer (pH 7·4) 25 μmol; mitochondrial suspension, 0·5 ml (equivalent to 1·8 g tissue). ADP (varying amounts up to 10 μmol—see Results section) dissolved in 0·2 ml water was placed in each of the side arms of the flasks and added as indicated. The "control" flask had 0·2 ml water in the side arms to be tipped in at the same time as the ADP. The final volume was made to 2 ml with water.

The same method was used for the mitochondria of Series B fruit except that, since in some experiments P:O ratios were measured directly on replicate samples as used in the respiratory control measurements, 0·5 μmol of NAD was also added to the contents of all the flasks. With both pulp and peel mitochondria, 1·0 ml of the suspension was used representing 9·1 g of pulp tissue and 4·55 g of peel tissue.

[17] R. J. L. Allen, *Biochem. J.* 34, 858 (1940).

Chemicals

All the chemicals used were the same as listed in paper I of the present series.[15] Glucose was Analar grade. Two sources of hexokinase were used, Sigma Chemical Co. type II, and Boehringer u. Soehne—crystalline analytical grade. Twice glass-distilled water was used throughout.

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